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Inhibition of glomerular cell apoptosis by heparin

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Inhibition of glomerular cell apoptosis by heparin.

Background. Heparin, the multifunctional glycosaminoglycan, has been considered a therapeutic agent for glomerular diseases. Although a number of biological properties are postulated to explain its therapeutic utility, it is unknown whether heparin affects cell survival in the glomerulus. In this report, we investigated the effect of heparin on apoptosis of glomerular cells.

Methods. Cultured rat mesangial cells were pretreated with heparin or heparan sulfate proteoglycan (HSPG) and were exposed to proapoptotic stimuli. To examine an effect of heparin on spontaneous apoptosis that occurs in explanted glomeruli, isolated rat glomeruli were incubated in the presence or absence of heparin. Apoptosis was evaluated by Hoechst 33258 staining, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, and agarose gel electrophoresis to detect DNA fragmentation. The effect of heparin on activator protein 1 (AP-1), a crucial mediator for oxidant-induced apoptosis, was examined by Northern blot analysis and a reporter assay.

Results. Heparin and HSPG inhibited apoptosis of mesangial cells triggered by hydrogen peroxide. It was associated with blunted expression of c-*fos*/c-*jun* mRNAs and suppression of AP-1 activation. The cytoprotective effect of heparin was also observed in other cell types and in apoptosis triggered by different stimuli. That is, (*a*) heparin inhibited mesangial cell apoptosis induced by staurosporine, pyrrolidine dithiocarbamate, and ultraviolet light, and (*b*) heparin suppressed oxidant-induced apoptosis of NRK49F fibroblasts and Madin-Darby canine kidney epithelial cells. Furthermore, heparin attenuated spontaneous apoptosis of podocytes in explanted glomeruli.

Conclusions. These results indicate the novel potential of heparin as an inhibitor of apoptosis in several cell types, including glomerular cells.

Heparin is generally described as an anionic polysaccharide or a sulfated glycosaminoglycan with irregular sequences. Heparin has a characteristic, anticoagulant property and is synthesized in various tissues, especially in the liver, lung, and gut. In the kidney, it has been shown that glomerular epithelial and endothelial cells have the ability to produce heparin-like glycosaminoglycans [1, 2].

Heparin has been used as a therapeutic agent for the treatment of glomerulonephritis. Systemic administration of heparin attenuates several experimental and human glomerular diseases, including antiglomerular basement membrane nephritis, Habu venom-induced injury, puromycin aminonucleoside nephrosis, remnant-kidney models, anti-Thy 1 glomerulonephritis, and lupus nephritis [3, 4]. The therapeutic action of heparin is ascribed to its anti-inflammatory properties, including inhibition of blood coagulation, complement inactivation, and suppression of leukocyte function [4]. For example, heparin inhibits leukocyte rolling and adhesion on the endothelium [5, 6], and aggregation, degranulation and superoxide anion generation by activated neutrophils [7]. In addition to these effects, heparin has direct actions on resident glomerular cells. Heparin and heparan sulfate proteoglycan (HSPG) inhibit mitogenesis and migration of cultured mesangial cells [1, 2, 8–10]. Heparin and HSPG inhibit induction of immediate early genes [11, 12] and attenuate the expression of potentially injurious mediators in mesangial cells [13]. Heparin and HSPG also inhibit proliferation of glomerular epithelial cells [14, 15] and protect endothelial cells from oxygen radical-mediated injury [16, 17].

Although a number of biological properties have been postulated to explain the therapeutic effects of heparin on glomerular disease, it is still unknown whether and how heparin modulates survival of glomerular cells. In this investigation, we address a novel potential of heparin for protecting glomerular cells and other cell types from apoptosis, which is initiated by several different stimuli.

METHODS

Cells and isolated glomeruli

Mesangial cells (SM43 cells) were established from isolated glomeruli of a male Sprague-Dawley rat and

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were identified as being of the mesangial cell phenotype as described before [18]. The kidney fibroblast cell line NRK49F and the tubular epithelial cell line Madin-Darby canine kidney (MDCK) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 100 U/ml of penicillin G, 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B, and 10% fetal calf serum (FCS).

Normal glomeruli were isolated from adult male Sprague-Dawley rats (250 to 300 g body wt) using the conventional sieving method [19]. We recently reported that resident cells, especially podocytes, spontaneously undergo apoptosis immediately after explantation of normal glomeruli [19]. For the induction of the spontaneous apoptosis, glomeruli were suspended in culture medium containing 1% FCS, incubated at 37°C for up to two hours in the presence or absence of heparin (0 to 100 U/ml; Sigma Immunochemicals, St. Louis, MO, USA) and subjected to agarose gel electrophoresis and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), as described later in this article.

Pharmacological manipulations

For the induction of apoptosis, confluent mesangial cells cultured in the presence of 1% FCS for 24 hours were treated by hydrogen peroxide (H_2O_2; 100 to 150 μM; Sigma), staurosporine (1.5 μM; Sigma), or pyrrolidine dithiocarbamate (PDTC; 50 μM; Sigma) for up to 24 hours. Irradiation of ultraviolet light (200 mJ) was also used to induce apoptosis of mesangial cells. Compared with mesangial cells and NRK49F cells, MDCK cells are relatively resistant to H_2O_2-induced injury. A higher concentration of H_2O_2 (350 μM) was used to induce apoptosis for this cell type.

To examine the effect of heparin and HSPG, cells were pretreated with heparin (5 to 100 U/ml; Sigma) or HSPG (500 μg/ml; Sigma) for 1.5 hours and were then exposed to proapoptotic stimuli. Heparin at the concentration of 100 U/ml was generally used for experiments. Heparin and HSPG were present in culture medium throughout exposure of the cells to apoptotic stimuli.

Trypan blue analysis

Viability of cells (both attached and detached cells) was assessed by trypan blue exclusion. After induction of apoptosis, cells were gently trypsinized and mixed with the same volume of 0.4% trypan blue solution (Sigma). Percentages of viable cells were evaluated by light microscopy. Assays were performed in quadruplicate.

Hoechst staining

Cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes, stained by Hoechst 33258 (10 μg/ml; Sigma) for one hour, and subjected to fluorescence microscopy. Apoptotic cells were identified by nuclear condensation and/or fragmentation. Percentages of apoptotic cells were evaluated quantitatively. When a substantial number of cells were detaching, detached cells and attached cells were evaluated separately, and total percentages of apoptotic cells were calculated.

TUNEL

TUNEL assay was performed using the Apoptosis Detection System, Fluorescein (Promega, Madison, WI, USA) [19]. In brief, cells and isolated glomeruli (5×10^3) were fixed by 4% paraformaldehyde in PBS overnight at 4°C. The samples were washed three times with PBS and were permeabilized by 0.2% Triton X-100 in PBS for 15 minutes on ice. After washing twice, cells and glomeruli were equilibrated at room temperature for 15 to 30 minutes in equilibration buffer (200 mM potassium cacodylate, 0.2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride in 25 mM Tris-HCl, pH 6.6) and then incubated in the presence of 5 μM fluorescein-12-dUTP, 10 μM dATP, 100 μM ethylenediaminetetraacetic acid (EDTA), and terminal deoxynucleotidyl transferase at 37°C for 1.5 hours in dark. The tailing reaction was terminated by 2 × standard saline citrate (SSC). The samples were washed three times with PBS and were analyzed by fluorescence microscopy.

Ladder detection assay

After induction of apoptosis, cells (5×10^5/sample, both attached and detached cells) and isolated glomeruli (2.5 to 5×10^3) were lyzed with 150 μl hypotonic lysis buffer (10 mM EDTA, 0.5% Triton X-100 in 10 mM Tris-HCl, pH 7.4) for 15 minutes on ice and were precipitated with 2.5% polyethylene glycol and 1 M NaCl for 15 minutes at 4°C. After centrifugation at 16,000 *g* for 20 minutes at room temperature, the supernatant was incubated in the presence of proteinase K (300 μg/ml; Sigma) at 37°C for one hour and precipitated with isopropanol at −20°C. After centrifugation, each pellet was dissolved in 10 μl of Tris-EDTA (pH 7.6) and electrophoresed on a 1.5% agarose gel containing ethidium bromide. Ladder formation of oligonucleosomal DNA was detected under ultraviolet light.

Northern blot analysis

Expression of c-*fos* and c-*jun* was examined by Northern blot analysis [20, 21]. In brief, mesangial cells were exposed to H_2O_2 (100 μM) for 0.5 to 2 hours in the presence or absence of heparin (5 to 100 U/ml) and were subjected to analysis. Total RNA was extracted by a

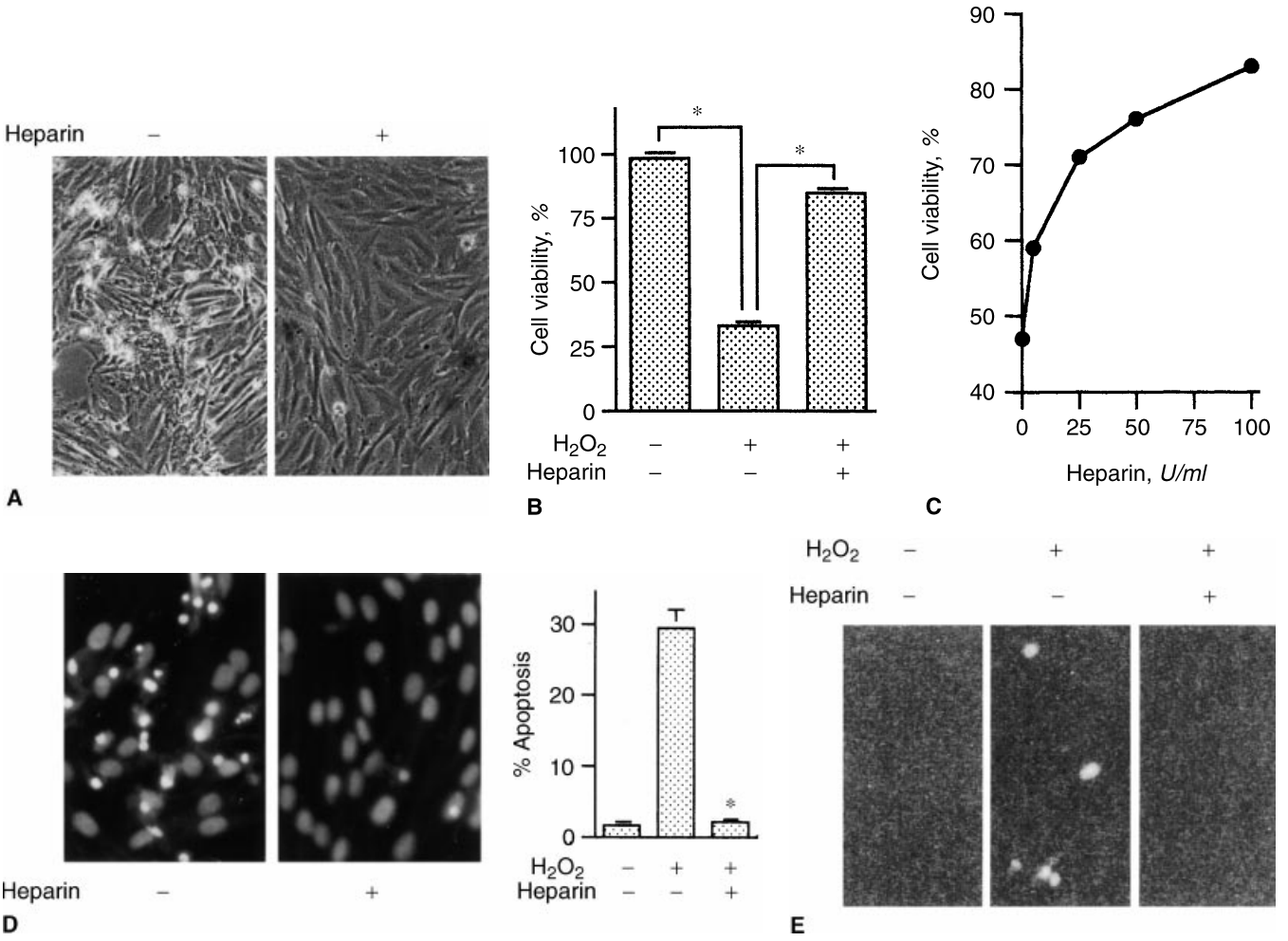

Fig. 1. Effect of heparin on oxidant-triggered apoptosis of mesangial cells. Confluent rat mesangial cells were pretreated with (+) or without (−) heparin (5 to 100 U/ml) for 1.5 hours in the presence of 1% fetal calf serum (FCS) and were exposed to hydrogen peroxide (H_2O_2; 100 to 150 μM) for up to 24 hours. Heparin 100 U/ml was generally used for experiments. (*A*) Phase-contrast microscopy: 150 μM H_2O_2 for 16 hours. (*B*) Trypan blue analysis. After induction of apoptosis (100 μM H_2O_2, 24 hr), mesangial cells were gently trypsinized and mixed with the same volume of trypan blue solution. Percentages of viable cells were evaluated by light microscopy. Assays were performed in quadruplicate. Data were expressed as means ± SE. Asterisks indicate statistically significant differences ($P < 0.05$). (*C*) Trypan blue analysis: dose-dependent effect. Cells were pretreated with 0 to 100 U/ml heparin, exposed to 150 μM H_2O_2 for 24 hours, and subjected to trypan blue analysis. (*D*) Hoechst staining. After induction of apoptosis (150 μM H_2O_2, 24 hr), cells were fixed and stained by Hoechst 33258 for one hour and subjected to fluorescence microscopy. Apoptotic cells were identified by nuclear condensation and/or fragmentation, and percentages of apoptotic cells were calculated. Assays were performed in quadruplicate. An asterisk indicates a statistically significant difference against H_2O_2-treated, heparin-untreated cells ($P < 0.05$). (*E*) Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). After induction of apoptosis (150 μM H_2O_2, 6 hr), cells were subjected to TUNEL, as described in the **Methods** section (fluorescence microscopy). (*F*) Ladder detection assay. Cells were pretreated with 0 to 100 U/ml heparin, exposed to 150 μM H_2O_2 for 16 hours, and subjected to agarose gel electrophoresis. (*G*) Effect of heparan sulfate proteoglycan (HSPG). Mesangial cells were pretreated with (+) or without (−) HSPG (500 μg/ml) for 1.5 hours in the presence of 1% FCS and exposed to H_2O_2 (150 μM) for 24 hours. Percentages of apoptotic cells were evaluated by Hoechst staining. An asterisk indicates a statistically significant difference ($P < 0.05$).

single-step method, and RNA samples were electrophoresed on 1.2% agarose gels and transferred onto nitrocellulose membranes. For hybridization, a human c-*fos* cDNA [22] and a human c-*jun* cDNA [23] were labeled with ^{32}P-dCTP using the random priming method. As a loading control, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. The membranes were hybridized with probes at 65°C overnight in a solution containing 4 × SSC (600 mM sodium chloride, 60 mM sodium citrate), 5 × Denhardt's solution, 10% dextran sulfate, 50 μg/ml herring sperm DNA, and 50 μg/ml poly(A), washed at 50°C and exposed to Kodak XAR films at −80°C.

Reporter assay

Activator protein 1 (AP-1) binds to the *cis* element, 12-*O*-tetradecanoate 13-acetate response element (TRE) and triggers transcription of target genes. To evaluate

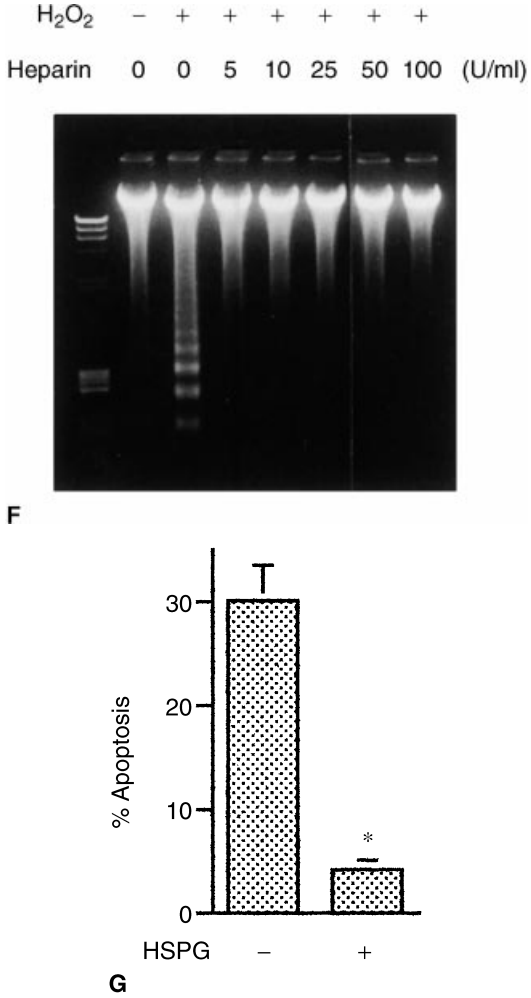

Fig. 1. Continued

the activity of AP-1 in mesangial cells, a reporter assay was used [24]. In brief, mesangial cells were cultured in 24-well plates (1.0 to 1.2 $\times$ 10^5/well). Using a calcium-phosphate coprecipitation method, the cells were transfected with a reporter plasmid pTRE-LacZ (0.3 to 0.4 μg/well) [25] that introduces a β-galactosidase gene (*lacZ*) under the control of TRE. After incubation for 48 hours in the presence of 1% FCS, the cells were pretreated with or without heparin for 1.5 hours and were stimulated by H_2O_2 (100 μM) for 24 hours. The cells were fixed and subjected to 5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside (X-gal) assay, as described previously [26]. Activity of AP-1 was evaluated by counting X-gal–positive cells in each well. Assays were performed in quadruplicate.

Statistical analysis

Data are expressed as means ± SE. Statistical analysis was performed using the nonparametric Mann–Whitney *U*-test to compare data in different groups. A P value of <0.05 was used to indicate a statistically significant difference.

RESULTS

Effect of heparin on oxidant-triggered apoptosis of mesangial cells

Mesangial cells undergo apoptosis following exposure to H_2O_2 [27]. To examine the effect of heparin on the oxidant-initiated apoptosis, mesangial cells were pretreated with or without heparin (100 U/ml) and were exposed to H_2O_2 (150 μM). Microscopic analysis showed that heparin protected mesangial cells from morphological apoptotic changes induced by the oxidant stress (Fig. 1A).

The apoptotic process is divided into three phases. In the first and second phases, the function of cellular membranes is retained intact, but in the third phase, cell membranes are progressively degenerated. In this last phase, secondary necrosis is observed as the final step of apoptosis [28]. To confirm that heparin indeed inhibits mesangial cell death, trypan blue analysis was performed. Compared with untreated control (cell survival 98.4 ± 0.2%, mean ± SE), H_2O_2 reduced the cell viability to 32.9 ± 2.8%. Pretreatment with heparin significantly improved the cell survival to 84.7 ± 0.5% ($P < 0.05$; Fig. 1B). The cytoprotective effect of heparin was dose-dependent. An obvious increase in the cell survival was observed at a low concentration (5 U/ml), but its maximum effect was achieved by the highest concentration tested (100 U/ml; Fig. 1C).

The suppressive effect of heparin on the mesangial cell apoptosis was confirmed by Hoechst staining and TUNEL. Staining of the cells with Hoechst 33258 exhibited condensation and fragmentation of nuclei in H_2O_2-exposed cells. It was markedly suppressed by the treatment with heparin; that is, the percentage of apoptosis was reduced from 29.5 ± 2.6% to 2.1 ± 0.2% by the treatment with heparin (Fig. 1D). Similarly, the number of TUNEL-positive cells was dramatically decreased by externally added heparin (Fig. 1E).

The anti-apoptotic effect of heparin was further confirmed by ladder detection assay. Agarose gel electrophoresis exhibited DNA ladder formation in H_2O_2-exposed mesangial cells. The DNA laddering was abrogated by the treatment with heparin (Fig. 1F). The lowest concentration (5 U/ml) of heparin was found to be sufficient to suppress the oxidant-induced DNA fragmentation.

To examine whether other heparin-like species also inhibit apoptosis of mesangial cells, the effect of HSPG was tested. Mesangial cells were pretreated with or without HSPG (500 μg/ml) for 1.5 hours and were exposed to H_2O_2 (150 μM). Hoechst staining showed that the

percentage of apoptotic cells was significantly reduced by the treatment with HSPG [percentages of apoptosis were 30.1 ± 3.4% in HSPG (−), and 4.2 ± 0.9% in HSPG (+); Fig. 1G].

Effect of heparin on the activation of activator protein 1, the mediator for oxidant-induced apoptosis

Activator protein 1 plays a crucial role in the H_2O_2-triggered apoptosis in mesangial cells [27]. We found that H_2O_2 induces expression of c-*fos*/c-*jun* and activation of AP-1 [27]. Down-regulation of c-Jun/AP-1 using either a dominant-negative mutant of c-*jun*, an antisense c-*jun*, or a pharmacological inhibitor of c-*jun* attenuated the H_2O_2-initiated apoptosis [27]. To elucidate mechanisms involved in the anti-apoptotic action of heparin, its effect on AP-1 was examined. Northern blot analysis showed that H_2O_2 induced expression of c-*fos* and c-*jun* in mesangial cells. Pretreatment with heparin abrogated the induction of these genes (Fig. 2A). Low concentrations (5 to 10 U/ml) of heparin were found to be sufficient to suppress the expression of c-*fos* and c-*jun* (data not shown). Consistently, transient transfection assay with a TRE reporter plasmid showed that the activation of AP-1 by H_2O_2 was abolished in the presence of heparin (Fig. 2B).

Effects of heparin on apoptosis of mesangial cells in response to different stimuli

To examine whether the cytoprotective action of heparin is restricted to apoptosis triggered by H_2O_2, other pro-apoptotic stimuli, including staurosporine and PDTC, were tested. Mesangial cells were pretreated with or without heparin and were exposed to staurosporine (1.5 μM) or PDTC (50 μM) for 24 hours. Hoechst staining showed that heparin significantly attenuated apoptosis in both experimental settings (Fig. 3 A, B). That is, the percentages of apoptotic cells were reduced from 34.6 ± 1.0% [heparin (−)] to 6.9 ± 0.2% [heparin (+)] in staurosporine-treated cells and from 12.1 ± 1.1% [heparin (−)] to 1.9 ± 0.2% [heparin (+)] in PDTC-treated cells.

The anti-apoptotic effect of heparin was further examined using ultraviolet light as a trigger. Mesangial cells were pretreated with or without heparin and subjected to ultraviolet irradiation (200 mJ). After 24 hours, Hoechst staining was performed. As shown in Figure 3C, heparin significantly attenuated the ultraviolet-induced apoptosis. The percentage of apoptotic cells was reduced from 17.0 ± 1.6% [heparin (−)] to 6.9 ± 0.9% [heparin (+)] by the treatment with heparin.

Effects of heparin on H_2O_2-initiated apoptosis in other cell types

To examine whether the anti-apoptotic effect of heparin against H_2O_2 is specific to mesangial cells, NRK49F fibroblasts and MDCK epithelial cells were tested. Dose-dependent effects of H_2O_2 on individual cell types were initially examined to determine minimum concentrations required for cellular damage. Compared with mesangial cells and NRK49F cells, MDCK cells were found to be relatively resistant to H_2O_2-induced injury. The minimum concentrations required were 150 μM for NRK49F cells and 350 μM for MDCK cells (data not shown). Using these concentrations, the effects of heparin on H_2O_2-induced apoptosis were examined.

NRK49F fibroblasts exposed to H_2O_2 exhibited shrinkage of the cytoplasm, membrane blebbing, and condensation of nuclei, like in mesangial cells (data not shown). Hoechst staining showed condensation and fragmenta-

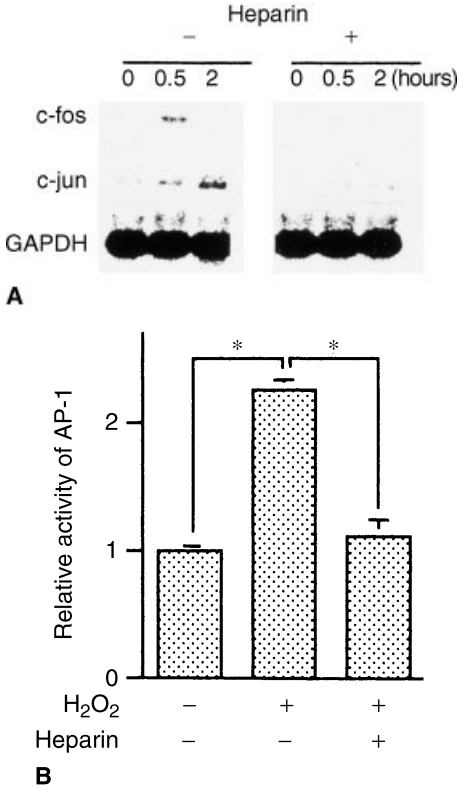

Fig. 2. Effect of heparin on the H_2O_2-triggered activation of activator protein 1 (AP-1). (*A*) Northern blot analysis. Mesangial cells were exposed to H_2O_2 (100 μM) for 0.5 to 2 hours in the presence (+) or absence (−) of heparin (100 U/ml) and were subjected to Northern blot analysis of c-*fos* and c-*jun*. As a loading control, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was shown on the bottom. (*B*) Reporter assay. Mesangial cells were transfected with a reporter plasmid pTRE-LacZ that introduces a β-galactosidase gene (*lacZ*) under the control of 12-*O*-tetradecanoate 13-acetate response element (TRE). After incubation for 48 hours in the presence of 1% fetal calf serum (FCS), the cells were pretreated with (+) or without (−) heparin for 1.5 hours and stimulated by H_2O_2 (100 μM) for 24 hours. The cells were fixed and subjected to 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) assay. Activity of AP-1 was evaluated by counting X-gal-positive cells in each well. Data are shown as a fold increase against the value of untreated control. Assays were performed in quadruplicate. Asterisks indicate statistically significant differences ($P < 0.05$).

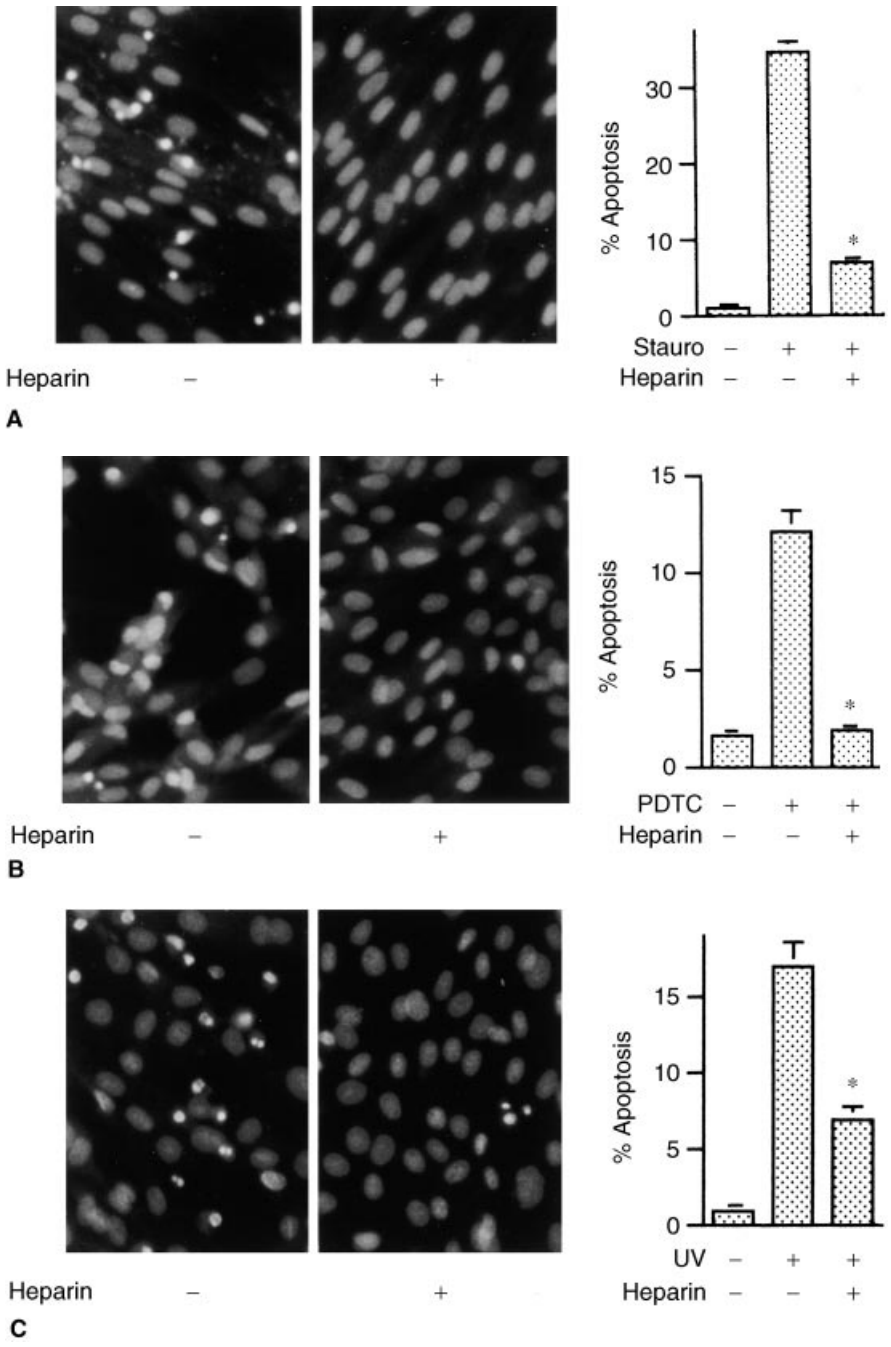

Fig. 3. Effects of heparin on apoptosis of mesangial cells triggered by different stimuli. Confluent mesangial cells were pretreated with (+) or without (−) heparin (100 U/ml) for 1.5 hours in the presence of 1% FCS and were exposed to (*A*) staurosporine (1.5 μM), (*B*) pyrrolidine dithiocarbamate (PDTC, 50 μM), or (*C*) ultraviolet light (UV, 200 mJ) for 20 to 24 hours. Cells were fixed, stained by Hoechst 33258, and subjected to fluorescence microscopy. Assays were performed in quadruplicate. Asterisks indicate statistically significant differences compared with the cells exposed to apoptotic stimuli without heparin ($P < 0.05$).

tion of nuclei in H_2O_2-exposed cells. Pretreatment with heparin significantly suppressed the apoptotic change (Fig. 4A). The percentage of apoptotic cells was reduced from 22.0 ± 1.4% [heparin (−)] to 1.1 ± 0.4% [heparin (+)]. Similarly, apoptosis of H_2O_2-exposed MDCK cells was attenuated by the treatment with heparin (Fig. 4B). The percentage of apoptotic cells was significantly reduced from 15.0 ± 0.4% [heparin (−)] to 3.3 ± 0.5% [heparin (+)]. Consistently, agarose gel electrophoresis showed that heparin inhibited DNA fragmentation in H_2O_2-exposed NRK49F cells and MDCK cells (Fig. 4C).

Effect of heparin on spontaneous apoptosis in explanted glomeruli

We recently found that glomerular cells, especially podocytes, spontaneously undergo apoptosis immediately after explantation of isolated glomeruli [19]. A number of resident cells are positive for TUNEL when

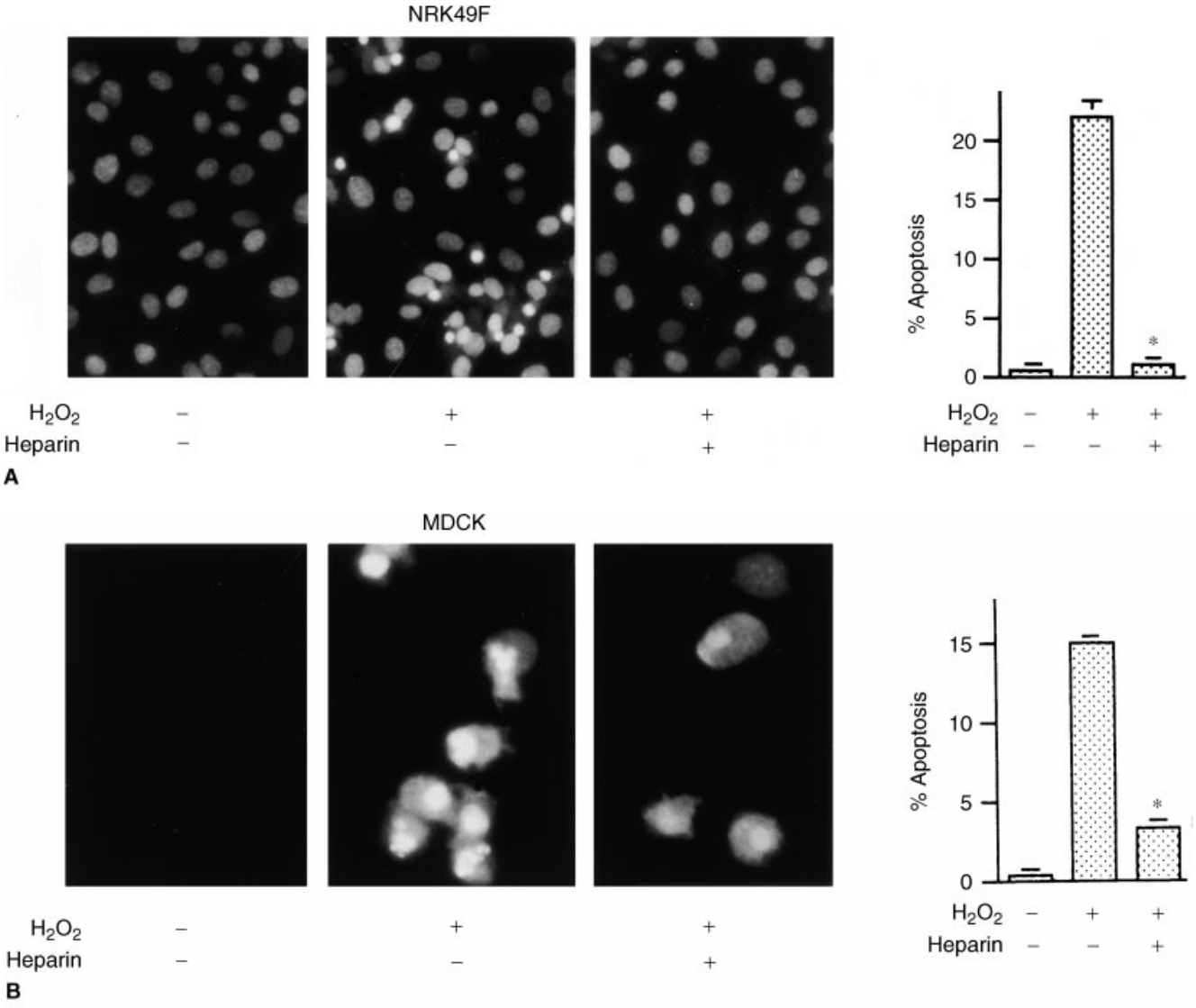

Fig. 4. Effects of heparin on H_2O_2-initiated apoptosis in other cell types. NRK49F fibroblasts and MDCK epithelial cells were pretreated with (+) or without (−) heparin (100 U/ml) for 1.5 hours in the presence of 1% FCS and were exposed to H_2O_2 (150 μM for NRK49F and 350 μM for MDCK) for 16 to 20 hours. Cells were then stained by Hoechst 33258 or subjected to ladder detection assay. Assays were performed in quadruplicate. Asterisks indicate statistically significant differences compared with H_2O_2-exposed, heparin-untreated cells ($P < 0.05$). (*A*) Hoechst staining of NRK49F cells (fluorescence microscopy). (*B*) Hoechst staining of detached MDCK cells. In contrast to mesangial cells and NRK49F cells, the majority of apoptotic MDCK cells detached from the culture plate. To evaluate apoptosis of MDCK cells quantitatively, detached cells and attached cells were stained by Hoechst 33258 separately, and total percentages of apoptotic MDCK cells were calculated. (*C*) Ladder-detection assay.

examined at two hours after the explantation. Ladder detection assay showed that within 15 minutes DNA fragmentation was detectable. DNA laddering was peaked at two hours and attenuated thereafter [19]. To examine whether heparin inhibits the spontaneous apoptosis of glomerular cells, isolated rat glomeruli were incubated in the absence or presence of heparin (5 to 100 U/ml) for two hours. Agarose gel electrophoresis revealed that heparin dose-dependently inhibited the DNA fragmentation observed in explanted glomeruli (Fig. 5A). Obvious inhibition was observed even at the lowest concentration (5 U/ml), but the maximum effect was achieved by higher concentrations (50 to 100 U/ml).

The effect of heparin on the spontaneous apoptosis was further confirmed by TUNEL assay. After incubation of glomeruli for 20 minutes in the absence of heparin, the number of TUNEL-positive cells was dramatically increased, as reported previously [19]. Treatment with heparin markedly diminished the number of apoptotic cells (Fig. 5B). The effect of heparin was further tested using longer incubation periods. When examined after incubation for 45 minutes and 2 hours, the suppressive effect of heparin on glomerular cell apoptosis was still observed. However, substantial numbers of glomerular cells became TUNEL positive even in the presence of 100 U/ml heparin (data not shown).

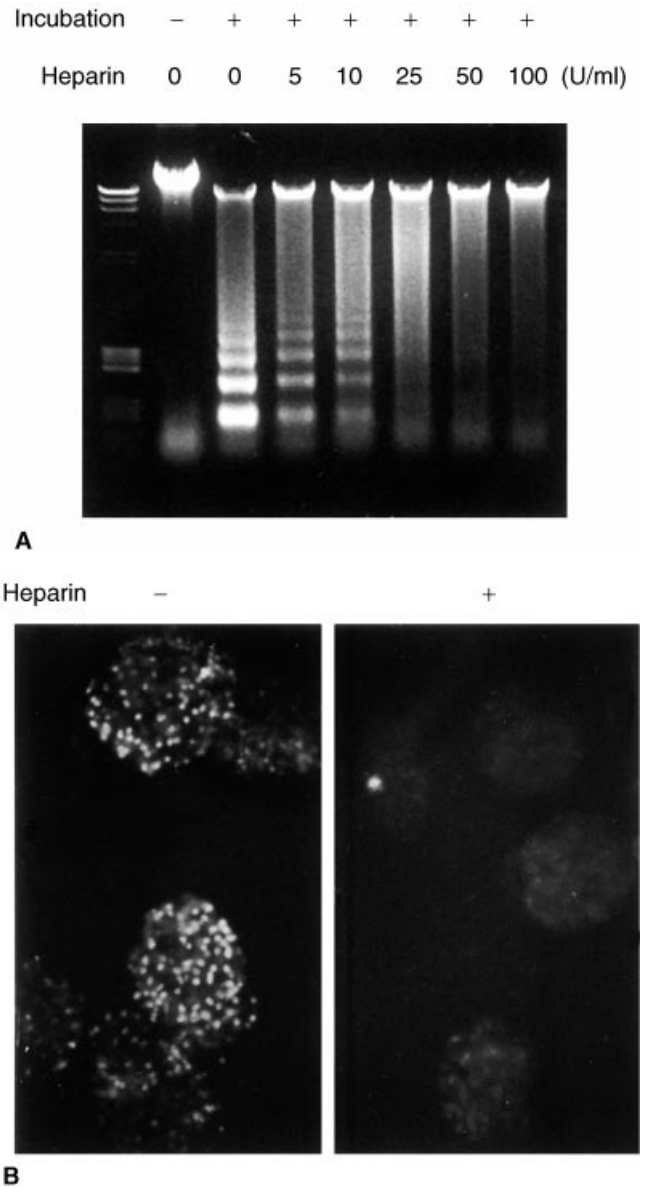

Fig. 5. Effect of heparin on spontaneous apoptosis in explanted glomeruli. (*A*) Ladder detection assay. Isolated, normal rat glomeruli were incubated at 37°C for two hours in culture medium containing 1% FCS and 0 to 100 U/ml of heparin and subjected to agarose gel electrophoresis. (*B*) TUNEL assay. Isolated glomeruli were incubated at 37°C for 20 minutes in the presence (+) or absence (−) of heparin (100 U/ml) and were subjected to TUNEL assay, as described in the **Methods** section. Glomeruli were examined by fluorescence microscopy.

DISCUSSION

Heparin has been considered an anti-mitotic agent for several cell types, especially for vascular smooth muscle cells and glomerular mesangial cells [3, 29]. In contrast, information is very limited regarding the effect of heparin on apoptotic processes. One previous report suggested that heparin may facilitate apoptosis in human peripheral blood neutrophils [30]. In our investigation, we demonstrated a novel potential of heparin as an inhibitor of apoptosis in glomerular cells. This study showed that heparin inhibits apoptosis in cultured mesangial cells and explanted glomeruli.

Molecular mechanisms involved in the antiapoptotic action of heparin are largely unknown. However, as demonstrated in this report, heparin attenuated H_2O_2-triggered expression of c-*fos* and c-*jun*. Reporter assays showed that heparin diminished activation of AP-1 in response to H_2O_2. These data suggested a possibility that heparin inhibits apoptosis of mesangial cells via intervention in the AP-1 pathway, the crucial signaling cascade involved in the oxidant-induced apoptosis [27]. The activity of AP-1 is regulated by both production of AP-1 protein and its activation via c-Jun N-terminal kinase (JNK). We examined the effect of heparin on the activity of JNK using c-Jun as a substrate. Immunoblot analysis showed that heparin did not attenuate activation of JNK in response to H_2O_2 (our unpublished observation). This result, together with our current findings, indicates a possibility that the inhibitory effect of heparin on the AP-1 pathway may be mainly through reduction in the mRNA and protein levels of AP-1 components.

The molecular targets for the anti-apoptotic action of heparin might be upstream of AP-1. Previous reports have shown that heparin facilitates the release of extracellular superoxide dismutase from endothelial cells, leading to their protection from oxygen radical-mediated

injury [16, 17]. Heparin could reinforce endogenous, antioxidant machinery and thereby inhibit the H_2O_2-induced apoptosis.

Heparin inhibited spontaneous apoptosis of podocytes in explanted glomeruli. The apoptosis was not inhibited by either catalase or antioxidant N-acetylcysteine when examined by ladder detection assay (our unpublished data). Furthermore, apoptosis of mesangial cells in response to the antioxidant PDTC was also inhibited by heparin. These results indicate a possibility that heparin interferes with a common pathway required for different apoptotic processes.

Apoptosis is observed in various glomerular diseases and may play a role in the initiation and progression of injury [31–34]. Previous studies suggested that therapeutic effects of heparin on glomerulonephritis are ascribed to its inhibitory actions on blood coagulation, complement function, and activation of both leukocytes and resident glomerular cells [35]. In addition to these putative mechanisms, our data imply another possibility that the therapeutic effect of heparin may be ascribed, in part, to its antiapoptotic property. It is worthwhile to note that a low concentration of heparin (5 U/ml) was found to be sufficient to attenuate inducible apoptosis of cultured mesangial cells as well as spontaneous apoptosis in isolated glomeruli.

Heparin-like species are widely distributed in the glomerulus, especially in the glomerular basement membrane and the mesangial matrix [36, 37]. The amount of HSPG in the glomerulus increases in experimental glomerular diseases including anti-Thy 1 glomerulonephritis, puromycin aminonucleoside nephrosis, and renal ablation models [38]. *In vitro*, glomerular cells produce heparin-like glycosaminoglycans. For example, glomerular epithelial and endothelial cells have the ability to secrete heparin-like molecules, which suppress mesangial cell proliferation [1, 2]. Mesangial cells and epithelial cells also produce HSPG that functions as an autocrine or paracrine growth inhibitor [15, 39, 40]. Moreover, as shown in this report, heparin and HSPG inhibit apoptosis of glomerular mesangial and epithelial cells. The intercellular network via heparin-like molecules might be one mechanism that protects glomerular cells from activation and injury.

As described in this report, heparin inhibited apoptosis not only in glomerular cells but also in other cell types, including renal fibroblasts and tubular epithelial cells. This finding suggests that heparin may function as a general inhibitor of apoptosis in a wide range of cell types. Further investigation will be required to determine the spectrum of the anti-apoptotic potential of heparin in various tissues and under various pathological situations.

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APPENDIX

Abbreviations used in this article are: AP-1, activator protein 1; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSPG, heparan sulfate proteoglycan; JNK, c-Jun N-terminal kinase; *lacZ*, β-galactosidase gene; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; TRE, 12-*O*-tetradecanoate 13-acetate response element; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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